Prognostic Biomarkers in Phase II Trial of Cetuximab-Containing Induction and Chemoradiation in Resectable HNSCC: Eastern Cooperative Oncology Group E2303

Amanda Psyrri1, Ju-Whei Lee2, Eirini Pectasides1, Maria Vassilakopoulou1, Efstratios K. Kosmidis6, Barbara A. Burtness3, David L. Rimm1, Harold J. Wanebo4, and Arlene A. Forastiere5

Abstract

**Purpose:** We sought to evaluate the correlation between tissue biomarker expression (using standardized, quantitative immunofluorescence) and clinical outcome in the E2303 trial.

**Experimental Design:** Sixty-three eligible patients with operable stage III/IV head and neck squamous cell cancer (HNSCC) participated in the Eastern Cooperative Oncology Group (ECOG) 2303 phase II trial of induction chemotherapy with weekly cetuximab, paclitaxel, and carboplatin followed by chemoradiation with the same regimen. A tissue microarray (TMA) was constructed and EGF receptor (EGFR), ERK1/2, Met, Akt, STAT3, β-catenin, E-cadherin, EGFR Variant III, insulin-like growth factor-1 receptor, NF-κB, p53, PI3Kp85, PI3Kp110a, PTEN, NRAS, and pRb protein expression levels were assessed using automated quantitative protein analysis (AQUA). For each dichotomized biomarker, overall survival (OS), progression-free survival (PFS), and event-free survival (EFS) were estimated by the Kaplan–Meier method and compared using log-rank tests. Multivariable Cox proportional hazards models were used to estimate HRs and test for significance.

**Results:** Forty-two of 63 patients with TMA data on at least one biomarker were included in the biomarker analysis. Tumor extracellular signal–regulated kinase (ERK)1/2 levels were significantly associated with PFS [HR (low/high), 3.29; \( P = 0.026 \)] and OS [HR (low/high), 4.34; \( P = 0.008 \)]. On multivariable Cox regression analysis, ERK1/2 remained significantly associated with OS \( (P = 0.024) \) and PFS \( (P = 0.022) \) after controlling for primary site (oropharynx vs. non-oropharynx) and disease stage (III vs. IV), respectively. Clustering analysis revealed that clusters indicative of activated RAS/MAPK/ERK and/or PI3K/Akt pathways were associated with inferior OS and/or PFS and maintained significance in multivariable analysis.

**Conclusions:** These results implicate PI3K/Akt and RAS/MAPK/ERK pathways in resistance to cetuximab-containing chemoradiation in HNSCC. Large prospective studies are required to validate these results.

Introduction

Locally advanced head and neck squamous cell cancer (HNSCC) may demonstrate resistance to therapy with concurrent chemoradiation, particularly in non-HPV–associated cancer, among smokers, and when disease is more advanced (1). Clinical trials of sequential chemotherapy and chemoradiation have not significantly altered cure rates (2, 3). It seems that even with aggressive sequential therapy programs, human papilloma virus (HPV)–negative patients have an extremely poor outcome. An important research strategy for poor prognosis HPV-negative cancers will be optimization of treatment regimens with the incorporation of novel active agents. In addition, the identification of biomarkers of resistance to chemoradiation is needed to select operable patients for surgery.

The first molecular targeting approach to demonstrate a survival advantage for patients with HNSCC has emerged in the context of EGF receptor (EGFR) biology. High cytoplasmic and nuclear EGFR protein level is associated with adverse outcome, and nuclear EGFR is highly associated with resistance to chemotherapy and radiation in HNSCC (4–6). Cetuximab is a chimeric immunoglobulin G1 (IgG1) human monoclonal antibody against the extracellular domain of EGFR, which exerts its antineoplastic properties by blocking ligand binding to the receptor. The clinical efficacy of cetuximab seems to involve multiple anticancer mechanisms, including inhibition of cell-cycle...
Translational Relevance
The objective of the present study is to evaluate the correlation between tissue biomarker expression (measured by automated quantitative protein analysis, AQUA) and clinical outcome in E2303, a phase II trial of induction chemotherapy with weekly cetuximab, paclitaxel, and carboplatin followed by chemoradiation with the same regimen in patients with operable stage III/IV head and neck squamous cell cancer (HNSCC). This AQUA assay allows measurement of protein expression within subcellular compartments to provide a value directly proportional to the number of molecules expressed per unit area. Patients who fail intensive chemoradiotherapy regimens are often not candidates for salvage surgery. Therefore, prognostic biomarkers for lack of response to aggressive sequential cetuximab-containing chemoradiation regimens may aid in the selection of patients for organ preservation strategies. We report that biomarker signatures consistent with activation of the RAS/MAPK/ERK/Pi3K pathways are inversely associated with outcome. In addition to prognostic information, our results indicate that targeting of the RAS or Pi3K pathways may restore sensitivity of HNSCC to cetuximab-containing chemoradiotherapy.

progression, apoptosis induction, inhibition of angiogenesis, inhibition of metastasis, and its ability to enhance the response to chemotherapy and radiotherapy (7). Cetuximab has been approved by the U.S. Food and Drug Administration (FDA) for locally advanced HNSCC in combination with radiation, and for recurrent/metastatic HNSCC in combination with platinum/5-fluorouracil chemotherapy, or as a single agent for patients who have failed platinum-based therapy (8, 9).

Eastern Cooperative Oncology Group (ECOG) 2303 studied the addition of cetuximab to a sequential program of weekly carboplatin and paclitaxel for 6 weeks, followed by cetuximab, carboplatin, and paclitaxel concurrent with radiation (Wanebo and colleagues; submitted for publication). Interim tumor assessment including biopsy was performed. We hypothesized that biomarkers of resistance to cetuximab and chemoradiation might be identified by scrutiny of EGFR and its dependent signaling intermediaries. EGFR activation triggers a signal transduction cascade that includes activation of the Ras/Raf/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (Pi3K)–Akt, and STAT (10–12).

Several candidate biomarkers for resistance to EGFR blockade in HNSCC have been proposed, but none has been validated (13–24). Immunohistochemical studies evaluating protein expression status in tumor samples are limited by inherent technical difficulties such as variability in antigen retrieval and immunohistochemical techniques, reduced reproducibility of pathologist-based scoring, and the semiquantitative nature of the assay. To overcome these problems, a method of in situ automated quantitative analysis (AQUA) has been developed, which allows measurement of protein expression within subcellular compartments to provide a value directly proportional to the number of molecules expressed per unit area (25). In this study, we used AQUA to evaluate protein expression of a panel of candidate prognostic biomarkers [EGFR, ERK1/2, Met, Akt, STAT, β-catenin, E-cadherin, EGFR Variant III (EGFRVIII), insulin-like growth factor-1 receptor (IGF-IR), NF-κB, p53, Pi3Kp85, Pi3Kp110a, PTEN, NRAS, and pRb], then further determine the correlation between biomarker expression and response to treatment in ECOG E2303 trial.

Materials and Methods
Cohort description
The ECOG 2303 sequential phase II trial of weekly cetuximab, carboplatin, and paclitaxel for 6 weeks, followed by the same combination concurrent with radiation focused on pathologic complete response, event-free survival (EFS), toxicity, and disease control (Wanebo and colleagues; submitted for publication). Sixty-three eligible patients were enrolled between December 15, 2004 and February 6, 2006. Paraffin-embedded specimens obtained for histologic diagnosis were collected after written informed consent was obtained. The protocol called for specimen collection at baseline, at week 8 (in complete clinical responders), and at week 14 (in those with persistent disease). Tumors from 45 patients, of whom 42 were eligible, were included in the tissue microarray (TMA) and subjected to biomarker analysis. All tumors included in the TMA were baseline biopsies as posttreatment biopsies were either negative or contained insufficient tumor for TMA construction. The TMA was constructed with 2-fold representation of each patient in two different masterblocks. The study complied with the REMARK recommendations for tumor marker prognostic studies using biologic material (available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2361579). A consort diagram is shown in Supplementary Fig. S1.

Quantitative immunofluorescence–based assessment of biomarkers
Hematoxylin and eosin–stained (H&E) slides were reviewed by the pathologist, evaluated for the presence of squamous cell carcinoma, and marked for 0.6-mm core extraction. Cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instruments) at the ECOG Pathology Center at Northwestern University. All tumors were represented with 2-fold redundancy. Cores from HPV18-positive HeLa and HPV16-positive Caski cell lines fixed in formalin and embedded in paraffin were selected for controls and included in the array. The TMA was then cut to yield 5-μm sections and placed on glass slides using an adhesive-tape transfer system (InstruMedics).

Slides were deparaffinized with xylene followed by ethanol. After rehydration in dH2O, antigen retrieval was accomplished by pressure cooking in 0.1 mol/L citrate buffer (pH, 6.0). Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in

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Translated from Greek: "Προσδιοριστικές Βιομάρκερ." Διαλεγμένες βιομάρκερ για αντίσταση στο EGFR σε θεραπεία με cetuximab και χημοραδιοθεραπεία έχουν προσφέρει επιχειρήσεις, χωρίς να έχουν επιβεβαιωθεί (13–24). Ημιμονικοχιατρικές έρευνες στην αξιολόγηση του προσδιορισμού της βιομάρκερς σε δείγματα πάτουν από απόδειξη τεχνικών δυσκολιών όπως η ποικιλομετρία στο αντιγονιακό διακόπημα και απομονωτικοχιατρικές τεχνικές, μειωμένη προσδιοριστικότητα των προσδιοριστικών κατακόρυφων αξιολόγησης, και το περιστατικό φαινόμενο του αντιγονιακού διακόπημα. Για να διαξιολογήσουμε αυτό το περιστατικό, έχουμε χρησιμοποιήσει το AQUA για την αξιολόγηση του προσδιορισμού του προσδιορισμού της βιομάρκερς σε επιδείκνυμες κομμάτια με το τακτικό προσδιορισμό της χημοραδιοθεραπείας σε περιστροφικούς με κεφαλή και βαθύ παγκόσμιο καρκίνο της ιστοσείρας και τουν ακρωτηριακού καρκίνου (HNSCC). Αυτό το AQUA assay επιτρέπει την αξιολόγηση του προσδιορισμού του προσδιορισμού της βιομάρκερς σε επιδείκνυμες κατακόρυφες αξιολόγησης που προέρχονται από επιδείκνυμες κομμάτια που περιέχουν τον θεραπεύοντα προσδιορισμό του EGFR. Αυτή η εξέλιξη επιτρέπει την αξιολόγηση του προσδιορισμού της βιομάρκερς σε επιδείκνυμες κατακόρυφες αξιολόγησης που προέρχονται από επιδείκνυμες κομμάτια που περιέχουν τον θεραπεύοντα προσδιορισμό του EGFR.
methanol for 30 minutes. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin (BSA) for 30 minutes at room temperature. Following these steps, slides were incubated with primary antibody at 4°C overnight. The antibodies used in this study are shown in Supplementary Table S1 along with the antibody names, vendors, and optimal titration used for each.

Subsequently, slides were incubated with goat anti-mouse or goat anti-rabbit secondary antibody conjugated to a horseradish peroxidase–decorated dextran polymer backbone (EnVision; Dako) for 1 hour at room temperature. Tumor cells were distinguished from stroma by use of anticytokeratin antibody cocktail (rabbit or mouse pan-cytokeratin antibody Z0622/M3515; Dako) with subsequent Alexa 546–conjugated goat anti-rabbit or goat anti-mouse antibody, respectively (A11010 or A11003; Molecular Probes). We added 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Target molecules were subsequently visualized with a fluorescent chromogen (Cy-5-tyramide; PerkinElmer). Slides were mounted with a polyvinyl alcohol–containing aqueous mounting media with anti-fade reagent (n-propyl gallate; Acros Organics). Appropriate positive controls were included in the TMA, including HeLa cell lines embedded in paraffin. For negative control, the primary antibody step was eliminated.

The AQUA method of quantitative immunofluorescence (QIF) was used to quantitatively measure the biomarkers that have been described previously (25). In brief, monochromatic, high-resolution images were obtained of each histospot after immunofluorescent staining as described herein. Images were captured by the PM-2000 microscope (HistoRx). We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. A tumor nuclei–specific compartment was created by using DAPI signal to identify nuclei, and the cytokeratin signal to define cytoplasm/membrane. The target signal (AQUA score) was expressed as pixel intensity divided by the target area (tumor nuclei compartment). AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor. The AQUA scoring was done blinded to clinical results.

Statistical analysis

The primary endpoint in E2303 was the 1-year post-treatment EFS rate, which was estimated by the proportion of patients alive and free of surgery to the primary site, or disease progression 16 months after registration (i.e., at 12 months after finishing 4 months of induction treatment and chemoradiation). Patients without documented events were censored at the time of the last disease evaluation.

Frequency and percentage were used to characterize patient demographics and disease characteristics. The Fisher exact test or the Wilcoxon rank-sum test was used to make comparisons between groups. The mean of the pretreatment biomarker AQUA scores was calculated for each patient. All biomarker measurements were log-transformed (log_{10}) before further data analysis. The logistic regression model was used to examine the relationship between biomarker measurements (treated as on a continuous scale) and objective response (CR+PR). The Cox proportional hazards (PH) model was used to evaluate the relationship between biomarker measurements (treated as on a continuous scale) and event–time distributions. This analysis was based on the eligible and treated patients with evaluable biomarker data.

Biomarkers with significant association with any efficacy outcome were further investigated. Patients were dichotomized into two groups at a series of cut points (the first quartile (Q1), the median (Q2), or the third quartile (Q3)) with respect to the biomarker AQUA scores. The Fisher exact test was used to evaluate differences in response rate between groups (high vs. low AQUA scores). Event–time distributions were estimated by the Kaplan–Meier method and compared by the log-rank test. The univariable and multivariable Cox PH models were used to evaluate the relationship between biomarker status (high vs. low AQUA scores) and event–time distributions, with age, sex, race (White vs. Black), performance status (0 vs. 1), weight loss in previous 6 months (<5% vs. ≥5%), stage (III vs. IV), cell differentiation grade (grade I/II vs. grade III/IV), and primary site (oropharynx vs. non-oropharynx), smoking status (never smoker vs. former smoker vs. current smoker) as potential covariates. Only covariates with a significant association (P < 0.10) with the event–time distribution in a univariate model were fitted into the multivariable model to further evaluate the biomarker. On the basis of our own preclinical data (Rampias and colleagues; submitted for publication; ref. 26), we subsequently investigated each of the following biomarker clusters with respect to overall survival (OS), progression-free survival (PFS), and EFS: (1) the combination of high PI3K85, high PI3K110, high NRAS, and low PTEN; (2) the combination of high PI3K85, high PI3K110, low akt, and low PTEN; and (3) the combination of high NRAS and low ERK. In this biomarker cluster analysis, the cutoff point for "high" is the upper quartile and the one for "low" is the lower quartile of each biomarker. Patients who could not be definitely classified into clusters due to data unavailable on the examined biomarkers were excluded from the data analysis.

Because the analysis of biomarker data was exploratory in nature, no statistical adjustments were made for tests on multiple biomarkers. All P values are two-sided. A level of P < 0.05 is considered statistically significant unless specified otherwise.

Results

Clinical and pathologic variable analysis

Table 1 shows details of patient demographic and disease characteristics at baseline. Among the 63 eligible patients who started protocol treatment, 42 eligible patients with TMA data on at least one biomarker were further analyzed and included in this report. The median
age of these 42 patients was 56 years (range, 30–72 years). Patients excluded from the analysis did not differ from those successfully evaluated by AQUA with respect to all parameters listed in Table 1. Supplementary Table S2 summarizes the median time and HR by ERK status. The multivariable Cox regression analysis shows that ERK is still significantly associated with OS [HR (low/high), 3.56; 95% CI, 1.20–10.55; P = 0.022] and PFS [HR (low/high), 3.56; 95% CI, 1.20–10.55; P = 0.022] after controlling for primary site and disease stage, respectively (Table 2). These results indicate that patients with higher ERK AQUA scores had a superior OS and PFS than those with lower ERK AQUA scores. Figure 1A–C display, respectively, the OS curves, PFS curves, and EFS curves by ERK status (high vs. low). On the basis of the continuous scale, pRb was marginally significantly associated with EFS (P = 0.051). On multivariable analysis, low pRb level (divided by the median) was a significant predictor for improved EFS (HR, 0.33; 95% CI, 0.11–0.98; P = 0.046) after adjusting for gender. HPV/E7 oncoprotein degrades pRb; therefore, low pRb might be a surrogate marker for HPV association. The 2-year and the 4-year OS were 0.046) after adjusting for gender. HPVE7 oncoprotein degrades pRb; therefore, low pRb might be a surrogate marker for HPV association. The 2-year and the 4-year OS rates, respectively, for pRb were 0.74 (high) and 0.88 (low) and 0.61 (high) and 0.69 (low). Figure 2A–C exhibit, respectively, the OS curves, PFS curves, and EFS curves by pRb status (high vs. low).

To understand the implications of ERK levels in the EGFR activation network, we used a computational model of the EGFR-induced MAP kinase cascade activation. The model is based on data from HeLa cells and describes the interaction of 94 components within the MAP kinase pathway in terms of coupled ordinary differential equations (27). The inverse relationship of the concentrations of ERK and its activated form, phosphorylated ERK (ERK-PP) is demonstrated in this model.

We subsequently performed clustering analysis including components of the RAS/MAPK/ERK and/or PI3K/Akt

### Survival analysis

E2303 (Wanebo H and colleagues; submitted for publication) reported a 95% primary site path complete remission (CR) in biopsied patients and 90% response in eligible patients with a 3-year OS of 78%, and EFS of 53%. p16 AQUA score, used as a surrogate marker for biologically and clinically meaningful HPV infection, did not emerge as a prognostic factor, probably due to the low prevalence of HPV in this HNSCC cohort.

Supplementary Table S3 lists ORs and HRs for association between the biomarker AQUA scores (on a continuous scale with a log10 transformation) and objective response (CR+PR vs. others), OS, PFS, and EFS, respectively. Except for the biomarkers of ERK and pRb, none of these examined biomarkers achieved statistically significant association with any efficacy outcomes. Specifically, the association between ERK and outcomes was noted in OS [HR, 0.13; 95% confidence intervals (CI), 0.03–0.53; P = 0.004] and PFS (HR, 0.25; 95% CI, 0.07–0.87; P = 0.03). Note HR (X) here refers to for every 1 unit increase (in the form of log10 transformation) in baseline biomarker expression, it is (1 – X)% less likely to experience the event. Thus, ERK was further examined with cutpoints at Q1, Q2, and Q3, separately. Because the cutpoint of Q1 produced the most significant results on the association between ERK and OS/PFS, Q1 was used for further comparison in OS and PFS between the two groups with high and low AQUA scores. Supplementary Table S3 summarizes the median time and HR by ERK status. The multivariable Cox regression analysis shows that ERK is still significantly associated with OS [HR (low/high), 3.61; 95% CI, 1.19–10.99; P = 0.024] and PFS [HR (low/high), 3.56; 95% CI, 1.20–10.55; P = 0.022] after controlling for primary site and disease stage, respectively (Table 2). These results indicate that patients with higher ERK AQUA scores had a superior OS and PFS than those with lower ERK AQUA scores. Figure 1A–C display, respectively, the OS curves, PFS curves, and EFS curves by ERK status (high vs. low). On the basis of the continuous scale, pRb was marginally significantly associated with EFS (P = 0.051). On multivariable analysis, low pRb level (divided by the median) was a significant predictor for improved EFS (HR, 0.33; 95% CI, 0.11–0.98; P = 0.046) after adjusting for gender. HPV/E7 oncoprotein degrades pRb; therefore, low pRb might be a surrogate marker for HPV association. The 2-year and the 4-year OS rates, respectively, for pRb were 0.74 (high) and 0.88 (low) and 0.61 (high) and 0.69 (low). Figure 2A–C exhibit, respectively, the OS curves, PFS curves, and EFS curves by pRb status (high vs. low).
ERK Pathway and Response to Cetuximab-Containing Therapy

Figure 1. Kaplan–Meier estimates of OS (A), PFS (B), and EFS by ERK status (high vs. low; C).
Figure 2. Kaplan–Meier estimates of OS (A), PFS (B), and EFS by pRb status (high vs. low; C).
ERK Pathway and Response to Cetuximab-Containing Therapy

Table 2. Univariable and multivariable Cox PHs regression analysis of OS/PFS/EFS by ERK and pRb (low vs. high status)

<table>
<thead>
<tr>
<th>Efficacy</th>
<th>Biomarker</th>
<th>Biomarker status</th>
<th>Number of events/N</th>
<th>Mediana (mon)</th>
<th>Log-rank test P</th>
<th>HR (95% CI; low vs. high)</th>
<th>P</th>
<th>HR (95% CI; low vs. high)</th>
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</thead>
<tbody>
<tr>
<td>OS</td>
<td>ERK1/2</td>
<td>Low</td>
<td>6/8</td>
<td>25.3</td>
<td>0.004</td>
<td>4.34 (1.47–12.83)</td>
<td>0.008</td>
<td>3.61 (1.19–10.99)</td>
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<td></td>
<td></td>
<td>High</td>
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<td>pRb</td>
<td>Low</td>
<td>5/16</td>
<td>—</td>
<td>—</td>
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<tr>
<td>PFS</td>
<td>ERK1/2</td>
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<td>15.4</td>
<td>0.019</td>
<td>3.29 (1.15–9.39)</td>
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<td>3.56 (1.20–10.55)</td>
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<td></td>
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<td>57.7</td>
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<tr>
<td>EFS</td>
<td>ERK1/2</td>
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NOTE: The high status and low status of ERK1/2 and pRb were determined by the first quartile and the median of each biomarker, respectively.

*aThe dash (—) indicates that the median time was not reached.

*bN = 34 for the ERK1/2 analysis; N = 32 for the pRb analysis. Primary site (oropharynx vs. non-oropharynx), disease stage (III vs. IV), and sex were fitted into the multivariable Cox PH models for OS, PFS, and EFS, respectively.

pathways. Results from univariate and multivariable Cox regression analysis by biomarker cluster are shown in Table 3 and Supplementary Figs. S2–S4. Clusters were statistically significantly predictive of differences in OS and/or PFS. Multivariable Cox regression analysis shows that (1) the combination of high PI3K85, high PI3K110, high NRAS, and low PTEN; (2) the combination of high PI3K85, high PI3K110, low Akt, and low PTEN; and (3) the combination of high NRAS and low ERK are playing important roles in predicting OS and/or PFS. Please refer to Table 3 for details.

Discussion

In the present study, we sought to determine biomarkers for resistance to cetuximab-containing chemoradiation in locally advanced HNSCC. The selection of patients for organ preservation approaches based on risk of recurrence is of critical importance. Efforts to define treatment-relevant subpopulations of HNSCC patients have been ongoing. The selection of biomarkers in this analysis was based on mechanistic insights gained from preclinical studies that implicate activation of downstream or alternative pathways in resistance to EGFR-targeted therapies. We identified ERK as a potential prognostic biomarker for resistance to the cetuximab-containing chemoradiation regimen studied in E2303 and found that low ERK protein levels were prognostic for shorter PFS and OS. ERK-PP is more frequently used to assess ERK activation status. However, routine formalin-fixed tissue does not provide the degree of preservation required to analyze functional proteins, particularly such transient events as phosphorylation (28). Data derived from computational biology studies indicate that there is an inverse association between ERK and ERK-PP. Therefore, low unphosphorylated ERK levels suggest that the activated phosphorylated ERK in cancer cells is high. We also found that biomarker signatures consistent with activation of the RAS/MAPK/ERK and/or PI3K/Akt pathways are associated with inferior outcomes to our cetuximab-containing chemoradiation regimen.

Activation of RAS/MEK1/2/ERK1/2 occurs frequently in malignancies and drives tumor cell proliferation, migration, and invasion by inducing antiapoptotic and proliferative pathways (29). ERK1/2 can also enhance the metastatic potential of tumor cells by inducing Slug, Snail, and epithelial-to-mesenchymal transition (EMT; ref. 30). ERK1/2 promote carcinogenesis in the context of PTPN13 loss in HPV-positive squamous cancers (31). In addition, studies in oral cavity squamous cancer cell line models from 7,12-dimethylbenz(a)anthracene–induced murine primary oral squamous cell carcinomas (OSCC) capable of tumor formation upon transplantation into immunocompetent wild-type mice showed that rapidly growing cell lines displayed ERK1/2 activation, which induced expression of CD44, a biomarker associated with EMT phenotype and putative cancer stem cells. MEK (MAP/ERK kinase) inhibition upstream of ERK1/2 reduced CD44 expression.

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Clin Cancer Res; 20(11) June 1, 2014
3029

Published OnlineFirst April 3, 2014; DOI: 10.1158/1078-0432.CCR-14-0113

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Akt pathways are resistant to chemoradiotherapy and activating activation of the RAS/MEK/ERK and/or PI3K/PI(3,4,5)P3 makes Akt a central mediator of cetuximab resistance in HNSCC, we have uncovered aberrant RAS/MAPK/ERK signaling as a central mediator of cetuximab resistance in HNSCC (Rampias and colleagues; submitted for publication). In addition, we found that there is molecular cross-talk between the RAS/MAPK/ERK and PI3K/akt pathways and that RAS is required for phosphoinositide 3-kinase (PI3K) activation in our head-and-neck cancer cell line and that RAS is required for phosphoinositide 3-kinase (PI3K) activation in our head-and-neck cancer cell line and that RAS is required for phosphoinositide 3-kinase (PI3K) activa

### Table 3. Univariable and multivariable Cox PHs regression analysis of OS/PFS/EFS by biomarker cluster

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<th>HR (95% CI; target vs. others)</th>
<th>P</th>
<th>HR (95% CI; target vs. others)</th>
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<td>OS</td>
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<td>0.30 (0.10–0.95)</td>
<td>0.041</td>
<td>0.29 (0.09–0.92)</td>
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<td>38.5</td>
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<tr>
<td></td>
<td>2</td>
<td>9/26</td>
<td>–</td>
<td>0.011</td>
<td>0.27 (0.10–0.79)</td>
<td>0.017</td>
<td>0.24 (0.08–0.69)</td>
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<td>Others</td>
<td>6/8</td>
<td>22.5</td>
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<tr>
<td></td>
<td>3</td>
<td>8/14</td>
<td>28.5</td>
<td>0.024</td>
<td>3.05 (1.10–8.45)</td>
<td>0.032</td>
<td>3.64 (1.28–10.33)</td>
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<tr>
<td></td>
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<td>–</td>
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<td>–</td>
<td>0.057</td>
<td>0.35 (0.11–1.09)</td>
<td>0.069</td>
<td>0.24 (0.07–0.83)</td>
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<td>35.4</td>
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<td>2</td>
<td>10/26</td>
<td>57.7</td>
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<td>0.024</td>
<td>0.25 (0.08–0.76)</td>
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<tr>
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<td>3</td>
<td>8/14</td>
<td>16.3</td>
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<td>2.14 (0.80–5.73)</td>
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<td>12/26</td>
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<tr>
<td></td>
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<td>8/14</td>
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<td>1.77 (0.71–4.43)</td>
<td>0.221</td>
<td>1.50 (0.59–3.81)</td>
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</table>

NOTE: The high and low status of the biomarkers reported in this table were determined by the upper quartile and the lower quartile of the biomarkers, respectively. Boldface indicates statistical significance.

^a The dash (–) indicates that the median time was not reached.

^b Primary site (oropharynx vs. non-oropharynx), disease stage (III vs. IV), and sex were fitted into the multivariable Cox PH models for OS, PFS, and EFS, respectively.

and promoter activity and inhibited cell migration and invasion (32). Basu and colleagues recently identified that tumor heterogeneity, likely because of EMT, establishes chemotherapy resistance in a mesenchymal-like subset of cells and that drugs that target this population are ideal candidates for future therapeutics (33). In our laboratory, by using a combination of cetuximab-resistant cell lines coupled with analyses of cetuximab-treated patients with HNSCC, we have uncovered aberrant RAS/MAPK/ERK signaling as a central mediator of cetuximab resistance in HNSCC (Rampias and colleagues; submitted for publication). In addition, we found that there is molecular cross-talk between the RAS/MAPK/ERK and PI3K/akt pathways and that RAS is required for phosphoinositide 3-kinase (PI3K) activation in our head-and-neck cancer cell line models. Others have also demonstrated that PI3Ks act as RAS effectors and these two pathways are interconnected rather than linear and independent (34, 35). The activated PI3K phosphorylates its substrate phosphatidylinositol biphosphate (PIP2) forming the secondary messenger PIP3, which recruits Akt into the plasma membrane and activates kinases (PDK1/PDK2) that, in turn, activate Akt. Our clustering analysis revealed that clusters indicative of RAS and/or PI3K activation are associated with poor outcomes. Therefore, head-and-neck cancer cells displaying activation of the RAS/MEK/ERK and/or PI3K/ Akt pathways are resistant to chemoradiotherapy and EGFR-targeted therapy.

These results, if validated, have important clinical implications. E2303 was conducted in operable locally advanced disease. Patients who fail intensive chemoradiotherapy regimens are often not candidates for salvage surgery. Therefore, prognostic biomarkers of resistance to aggressive sequential treatment programs including cetuximab may aid in the selection of patients for organ preservation strategies. We can also speculate that targeting of the RAS or PI3K pathways can restore sensitivity of HNSCC to cetuximab-containing chemoradiotherapy. Although MEK inhibitors have shown substantial toxicity in clinical trials, including blurred vision and neurotoxicity, novel compounds are being developed that may have reduced toxicity. PI3K inhibitors may also prove useful in the treatment of patients with HNSCC. A phase I/II study testing combined Mek and Akt inhibition in a variety of solid tumors including HNSCC is ongoing (a study of the safety and activity of E2303 in patients with multiple myeloma or solid cancers NCT01476137).
biomarkers. However, the selection of biomarkers was predetermined and the design was based on the assumption that more than 72 tumor samples would be included. The correlative study protocol had been designed as a pharmacodynamic evaluation at baseline, after induction and after chemoradiation. Unfortunately, the high response rate to induction did not allow biomarker evaluation on posttreatment biopsies. In addition, only histospots containing >5% of tumor cells were considered sufficient for AQUA analysis, further limiting the number of analyzable tumors.

In summary, our results indicate that biomarker signatures consistent with activation of the RAS/RAF/MEK/ERK/PI3K pathways are inversely associated with outcome in this ECOG phase II study of weekly induction chemotherapy plus cetuximab followed by cetuximab chemoradiotherapy. The evaluation of the prognostic value of these biomarkers in terms of cetuximab resistance needs to be evaluated in large randomized studies comparing chemotherapies plus/minus cetuximab.

Disclosure of Potential Conflicts of Interest
B.A. Burtens is a consultant/advisory board member for Boehringer Ingelheim and Bristol-Myers Squibb. D.L. Rimm is a consultant/advisory board member for Genoptix. No potential conflicts of interest were disclosed by the other authors.

References

26. Burtness BA, Yang Donghua, Zhu Fang, Garcia Joaquin J, Forastiere Arlene A, Chung Christine H. Activity of cetuximab (C) in head and neck squamous cell carcinoma (HNSCC) patients (pts) with PTEN loss or PIK3CA mutation treated on ES397, a phase III trial of cisplatin (DDP) with placebo (P) or C. J Clin Oncol 31, 2013 (suppl; abstr 6028).